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PRINCIPAL INVESTIGATOR:
Jason D. Weber, Ph.D.

CONTRACTING ORGANIZATION:
Washington University School of Medicine
St. Louis, MO 63110

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14. ABSTRACT
In this first annual review, we have demonstrated a clear role for the ARF tumor suppressor in regulating ribosome biogenesis in primary mouse mammary epithelial cells. Loss of ARF results in tremendous gains in rDNA transcription and rRNA processing. This mouse will be monitored over the next year for formation of breast tumors. We have also determined that ARF induction in the absence of the Tsc1 tumor suppressor is primarily achieved through increased translation of existing ARF mRNAs. We also made significant progress in our evaluation of p68 as a novel breast cancer oncoproteins that is suppressed by ARF. We have constructed a transgenic mouse overexpressing the ARF target p68 in the breast epithelium. We have shown that p68 causes increased ribosome biogenesis and that its expression is both required for the maintenance of proliferation in breast cancer cell lines and required for the transforming effects of oncogenic Ras in the absence of ARF.

15. SUBJECT TERMS
Cell growth, breast cancer cells, p68DDX5, ribosomes

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INTRODUCTION

Cancers result from an inability of a cell to control its own growth. Normally, a cell interprets external and internal signals to create a balanced growth schedule. The main interpreters of these signals within a cell are called ARF and p53, and it falls on the shoulders of these two proteins to maintain normal cell growth. In this sense, both ARF and p53 are tumor suppressors that constantly monitor the growth state of the cell. In mouse and human cancers, loss of the ARF tumor suppressor is second only to mutation of p53, providing critical evidence of ARF's role in both monitoring and preventing the outbreak of cancer cells. A common target of ARF is the NPM/B23 oncogene, an abundant protein of the nucleolus. NPM normally responds to growth factors and, due to its nucleolar localization, is thought to transmit these growth signals to the maturing ribosome machinery. Cells lacking *Arf* exhibit tremendous gains in ribosome production and subsequent protein synthesis. Moreover, the entirety of this growth phenotype is dependent on NPM and p68DDX5 expression in the nucleolus, with loss of either capable of completely reversing the phenotype back to normal. This exciting new finding indicates that ARF is a master regulator of cell growth through its tight control of NPM- or DDX5-directed ribosome production and export. Importantly, we have found NPM overexpressed in nearly 50% of breast carcinomas that we have analyzed, implying that dysregulation of NPM may be a key event in promoting breast cancer development. In effect, tumor cells that require increased protein synthesis might accumulate more NPM or DDX5 in an attempt to increase ribosome output. It is our goal to determine whether NPM directly regulates ribosome maturation to promote breast cancer formation and to establish the importance of ARF in deterring this effect. We propose to now determine the complex roles of ARF, DDX5, and NPM in the nucleolus of breast epithelial cells and how they impact both ribosome biogenesis and cell growth to prevent and/or promote tumorigenesis.

This work has tremendous clinical implications as *Arf* (9p21) and *p68DDX5* (17q24) reside on loci that are either deleted or amplified in ER+ resistant breast tumors, respectively. This fact makes our basic science on this interesting growth network directly applicable to the breast cancer phenotype/genotype.

BODY

As stated in the approved Statement of Work, we focused our energies on the tasks planned for Months 1-12. These included experiments outlined in Tasks 1 and 2. In this first Annual Progress Report, we detail the progress and results from these studies.

Task 1. Determine the role of ARF in suppressing breast tumor formation (Months 1-30):

a. Establish cultures of mouse mammary epithelial cells (MEC) from wild-type and *Arf*-null female virgin mice (Months 1-6). 50 mice per year.
b. Measure ribosome DNA transcription and rRNA processing in *Arf*-/- MEC (Months 6-18).

During the first year, we have concentrated our efforts into establishing a cell culture model for loss of *Arf* function in MMECs. To this extent, we now have immortal *Arf*-/- MMECs that retain a diploid and genomically stable genotype. They are still sensitive to DNA damage pathway activation events such as UV and gamma irradiation. Using these cells, we have now begun our studies of ribosome biogenesis pathway activation in the absence of *Arf*. We have discovered that MMECs lacking *Arf* exhibit extremely high levels of 47S transcription (**Figure 1**, top band). Additionally, *Arf*-null MMECs process rRNA at a considerably higher rate than wild-type counterparts, indicating that rRNA processing rates are elevated in the absence of *Arf* (Figure 1).

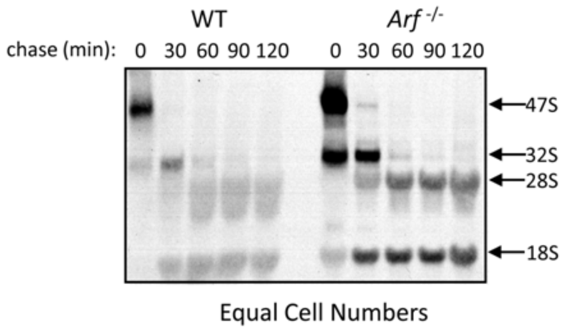

Figure 1. Ribosome DNA transcription and rRNA processing are enhanced in the absence of *Arf*. Primary MMECs harvested from wild-type (WT) and *Arf*-/- littermates were cultured in the presence of [^{3}H-methyl]-methionine for 30 minutes to allow for the labeling of the 47S rRNA transcript. Cells were then washed and cultured in label-free media for the indicated times to chase the label into processed rRNAs as indicated (Task 1b).

In the next year, we will build on this initial result to determine the mechanism for increased rDNA transcription and rRNA processing in MMECs lacking *Arf*. With this result in hand earlier than initially projected, we were able to begin some preliminary work on identifying the signaling pathway responsible for ARF induction.

f. Identify the signaling pathway(s) responsible for enhanced ARF mRNA translation (Months 12-24)

Loss of *Tsc1* results in hyperactivated mTOR signals, much the same as loss of *Pten* in primary MEFs. We first shifted to primary mouse embryonic fibroblasts (MEFs) because we had already previously established this cell type from *Tsc1*$^{fl/fl}$ animals that were sent to us by Dr. David Kwiatkowski. Additionally, we have been unable to establish cultured MMECs from *Tsc1*$^{fl/fl}$ animals due to technical difficulties in generating an efficient adenoviral Cre infection to effectively remove *Tsc1*. In the next year, we hope to overcome this difficulty and establish MMECs from these animals. When cultured in the presence of Cre-recombinase (adenoviral), *Tsc1* is excised from both alleles. In this setting ARF protein is induced in a delayed mechanism (**Figure 2**). This is agreement with our revised Statement of Work where I clearly stated that we would utilize RNA interference to lower PTEN or TSC1 to induce an ARF response. We had the *Tsc1*$^{fl/fl}$ animals, so that is where we began. Knockdown for both of these genes has been weaker that 50%, prompting us to utilize a Cre-lox system instead.

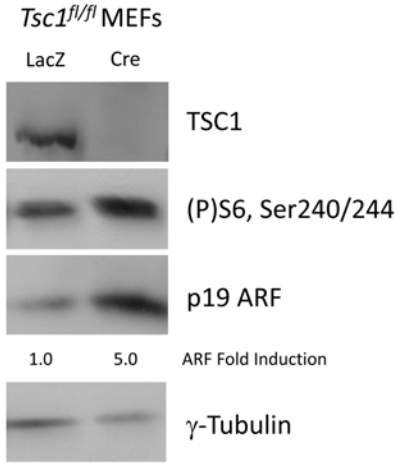

Figure 2. ARF protein is induced in the absence of *Tsc1*. Primary mouse embryonic fibroblasts from *Tsc1*$^{fl/fl}$ mice were cultured and infected with adenoviruses encoding LacZ or Cre recombinase for nine days. Cell pellets were lysed and proteins analyzed by western blot using antibodies recognizing TSC1, phospho-S6, ARF and gamma tubulin. ARF fold induction was determined by taking the ratio of ARF expression over tubulin control expression in LacZ versus Cre treated cells (Task 1f).

To determine the mechanism for ARF protein induction, we assayed the induction of ARF mRNA transcription following loss of *Tsc1*. Nine days following LacZ or Cre recombinase infection, cells were harvested and RNA was isolated. Northern blot analysis using probes specific for *Arf* exon 1β indicated that ARF mRNA expression was not increased upon loss of *Tsc1* (**Figure 3**), suggesting that any increase in ARF protein, as observed in Figure 2, was not due to an increase in ARF mRNA transcription.

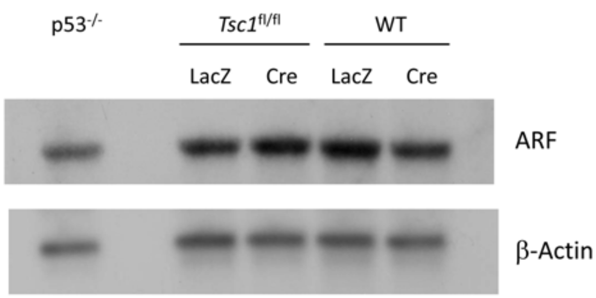

Figure 3. ARF mRNA transcription is not altered in the absence of *Tsc1*. Wild-type and *Tsc1*$^{fl/fl}$ MEFs were infected with LacZ or Cre adenoviruses for nine days. Total RNA was isolated from harvested cell pellets and separated on formaldehyde-agarose gels. Northern blot analysis using radio-labeled probes specific for murine *Arf* exon 1β revealed no change in ARF mRNA expression. Beta-Actin was used as a control (Task 1f).

Given that ARF mRNA transcription was not altered in the absence of *Tsc1*, we sought to determine a post-transcriptional mechanism for ARF protein induction. *Tsc1*$^{fl/fl}$ MEFs were radio-labeled with ^{35}S-methionine for various amounts of time, following a nine day infection with LacZ or Cre adenoviruses, to determine the rates of ARF translation. In the absence of *Tsc1* (+Cre), ARF translation results in visible protein accumulation by 0.75 hours after radio-label addition compared to 4 hours for control cells (**Figure 4**), indicating that ARF translation occurs at least 5 times faster in the absence of *Tsc1*.

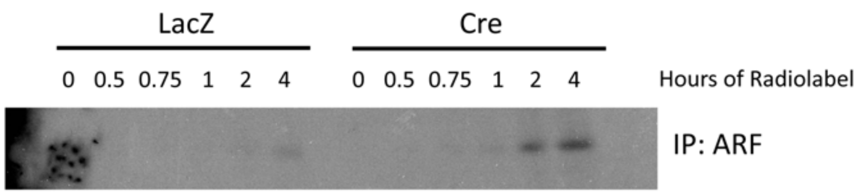

Figure 4. ARF translation is altered in the absence of *Tsc1*. *Tsc1*$^{fl/fl}$ MEFs infected with LacZ or Cre adenoviruses were pulse-labeled with ^{35}S-methionine for the indicated times. Lysates were immunoprecipitated with ARF monoclonal antibodies and Protein G Sepharose. Precipitated ARF proteins were separated by SDS-PAGE and visualized by autoradiography (Task 1f).

In the next year, we will strive to determine mechanism behind enhanced ARF translation in the absence of *Tsc1* using modified MMECs.

Task 2. Examine the mechanism behind NPM's ability to promote ribosome biogenesis and cell growth in breast epithelial cells (Months 1-36):

a. Determine the influence of NPM on ribosome biogenesis (Months 1-20). 50 mice per year.

To begin this Task, we have generated a transgenic mouse overexpressing a Histidine-tagged NPM protein in mammary epithelial cells. This transgenic mouse contains a transgene Lox-stop-Lox (LSL)-His-NPM on a mixed background (C57Bl6/129). These mice were crossed into MMTV-Cre mice to generate LSL-His-NPM/MMTV-Cre mice that will overexpress a histidine-tagged NPM in mammary tissues. **These mice were not generated with funds from this grant.** We will utilize MMECs isolated from these mice to complete Task 2a as stated in our Statement of Work.

Due to our rapid progress with Task 1, we were able to move forward with some preliminary aspects of Task 3.

Task 3. Establish the oncogenic potential of the p68DDX5 RNA helicase (Months 24-48):

a. Determine whether p68 is required for ribosome biogenesis (Months 24-36).

In order to perform this task, we first wanted to establish the role for p68 in cell growth and proliferation using MEFs and established breast cancer cell lines. Once these data are in hand, we will be able to move forward with this task in years 2 and 3. We now know that overexpression of p68 causes an increase in both rDNA transcription (**Figure 5A**) and in rRNA processing (**Figure 5B**), underscoring its importance in promoting ribosome biogenesis.

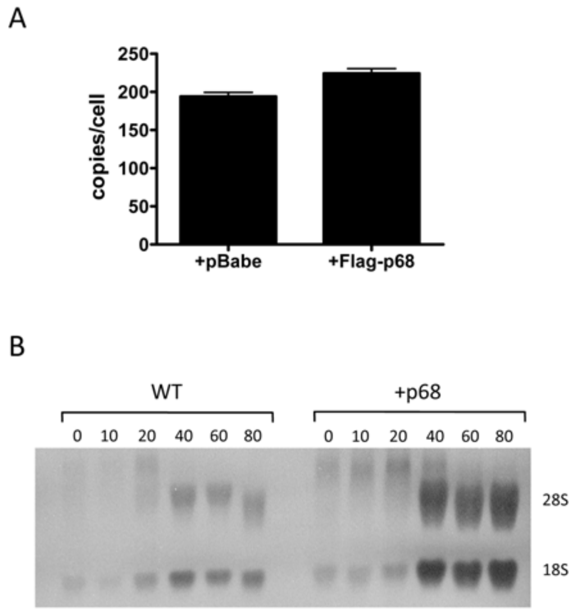

Figure 5. Ribosome DNA transcription and rRNA processing are increased in response to p68 overexpression. (A) Wild-type (WT) MEFs were infected with control pBabe or Flag epitope-tagged p68 overexpressing retroviruses and harvested 48 hours post-infection. Levels of rDNA transcription were measure as levels of 47S rRNA transcript by real-time PCR and are presented as copies per cell based on normalized inputs of control 47S expression plasmid. (B) MEFs infected with pBabe or p68 overexpressing retroviruses were cultured in the presence of [^{3}H-methyl]-methionine for 30 minutes to allow for the labeling of the 47S rRNA transcript. Cells were then washed and cultured in label-free media for the indicated times to chase the label into processed rRNAs as indicated (Task 3a).

We next sought to determine whether p68 expression is required for breast cancer cell transformation. We used the ER- breast cancer cell line HCC1806 which expresses high levels of 68 without amplifying the *DDX5* locus. We designed short interfering RNAs targeting the 3’ region of p68 mRNA in order to perform RNA interference. Infection of HCC1806 cells with lentiviruses encoding p68 knockdown hairpins demonstrated our ability to effectively lower endogenous p68 levels in these cells (**Figure 6**, top gel). Moreover, when we plated these infected cells in a limiting dilution of 5,000 cells per 100 mm, we observed a sharp decrease in cellular proliferation as judged by a lack of dividing cell foci (**Figure 6**, lower panels). Our findings indicate that in this breast cancer cell line, p68 expression is required for cell proliferation. In the next year, we will expand these results to include several more breast cancer cell lines and to test their tumorigenicity in animals.

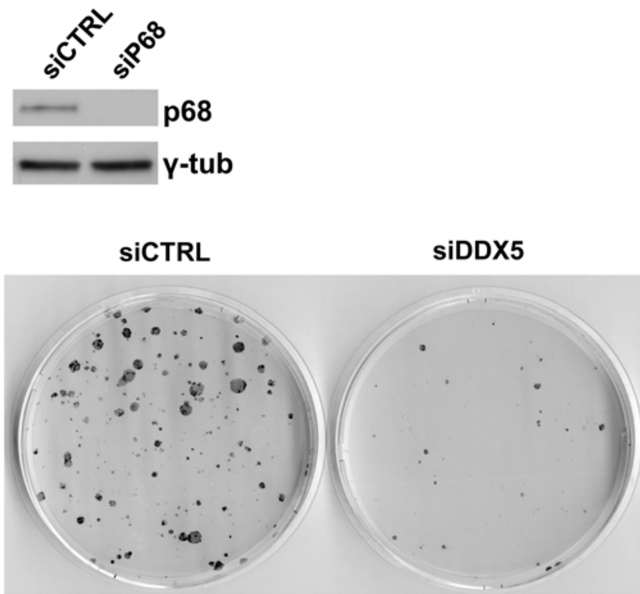

Figure 6. p68 expression is required for HCC1806 cell proliferation. HCC1806 cells were infected with lentiviruses encoding siRNAs directed against the 3' of human p68. Western blot analysis 48 hours post-infection confirmed effective p68 protein knockdown compared to gamma tubulin controls (upper panel). Infected cells were plated (5,000) onto 100 mm dishes and culture for 10 days. Resulting proliferative colonies were fixed in methanol and stained with Giemsa stain for visualization (Task 3c).

Having shown a role for p68 in the maintenance of the transformed phenotype of HCC18068, we sought to move this type of experiment into a more genetically defined cell background. We infected *Arf*-/- MEFs with lentiviruses encoding p68 siRNAs and then re-infected these same cells 24 hours later with retroviruses encoding oncogenic RasV12. In the absence of *Arf*, RasV12 readily transformed cells as depicted by their ability to grow in soft agar (**Figure 7**). However, in cells where p68 expression was greatly reduced with siRNAs against p68, RasV12 failed to transform and cells did not grow in soft agar (**Figure 7**). These data demonstrate a potentially novel role for p68 in assisting oncogenic Ras in the transformation of *Arf*-deficient cells. We will follow up on this exciting result next year using primary *Arf*-/- MMECs to determine the mechanism of action between Ras and p68.

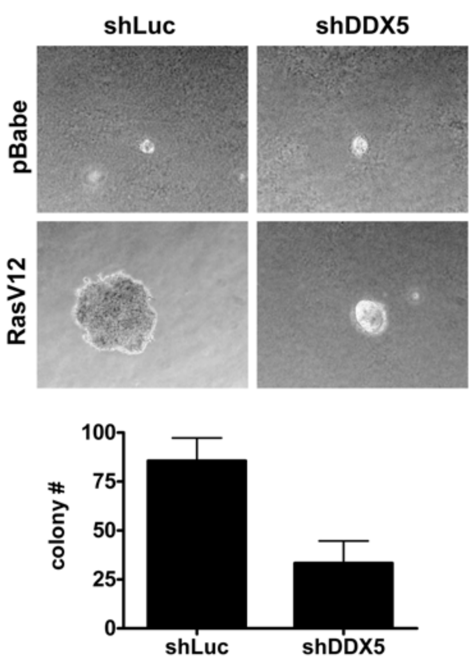

Figure 7. p68 expression is required for RasV12-mediated cellular transformation. *Arf* -/- MEFs were infected with lentiviruses encoding siRNAs targeting the 3' region of mouse p68. These cells were infected 24 hours later with retroviruses encoding oncogenic RasV12. Twenty-four hours post-infection, cells were plated (3,000) into soft agar containing DMEM and 10% FBS. Soft agar plates were re-fed every 3 days and visualized at 10X microscopy 21 days later. Multi-cell colonies were counted in three separate fields by three individuals and graphed with error bars representing standard deviations n=3 (lower panel) (Task 3c).

KEY RESEARCH ACCOMPLISHMENTS

- *Arf*-null mouse mammary epithelial cells (MMECs) are spontaneously immortal in culture (Task 1a)
- *Arf*-null MMECs are diploid and genomically stable (Task 1a)
- rDNA transcription is elevated in the absence of *Arf* (Task 1b)
- rRNA processing is enhanced in the absence of *Arf* (Task 1b)
- ARF mRNA is not induced by mTOR pathway activation (Task 1f)
- ARF translation is altered in the absence of *Tsc1* (Task 1f)
- p68 is required for breast cancer cell growth and proliferation (Task 3c)
- p68 increases ribosome production (Task 3a)
- p68 is required for RasV12-induced transformation (Task 3c)

REPORTABLE OUTCOMES

Manuscripts: None

Abstracts/Presentations: "Understanding and targeting cell growth networks in breast cancer" presented at the Era of Hope Conference in February 2009.

Patents/Licenses: None

Animal Models: We have generated a novel mouse model in the first year of this grant. The model is an *Arf* $^{fl/fl}$ mouse on a pure C57Bl6 background that was mated to a mixed MMTV-Cre mouse. The resulting *Arf* $^{fl/fl}$/MMTV-Cre mouse lacks ARF expression in mammary tissues. Once published, these mice will be available for free to any researcher that requests them.

Cell Lines: We have developed a unique primary mouse mammary epithelial cell (MMEC) line lacking the ARF tumor suppressor. These were established directly from *Arf* knockout mice on a pure C57Bl6 background. The *Arf*-null MMECs maintain a diploid phenotype and wild-type p53. These cells are spontaneously immortal and contain no artificial genes or plasmid constructs.

Funding Applied for: None

CONCLUSION

During the first year of funding, we have made significant progress on the Tasks outlined for Months 1-12. Due to our rapid progression, we have also begun Tasks for Months 12-24 regarding p68 function.

We have shown that *Arf*-null MMECs are immortal in cell culture models and that these cell lines do not contain aberrant chromosome numbers or alterations. They also maintain wild-type p53 and are quite capable of responding to typical p53-related stresses including gamma irradiation and hypoxia. These findings indicate that loss of *Arf* alone is sufficient to immortalize primary MMECs. These cells will prove to be a valuable tool to the general breast cancer research field.

In addition, we have shown that basic properties of ribosome biogenesis are altered in the absence of functional *Arf*. rDNA transcription and rRNA processing are both increased in the absence of basal ARF proteins, suggesting that ARF acts as a critical thermostat for ribosome levels in the cell. These results also argue that aberrant ribosome biogenesis might itself cause epithelial cell immortalization. This idea will be tested by my lab in the coming year.

We have also made significant progress in understanding the regulation of ARF levels in the cell. In the presence of hyperactive mTOR signaling, ARF protein is slowly induced. We now have shown that this induction is not due to increased transcription of ARF but rather in enhanced translation of existing ARF mRNAs. The question remains as to why this process occurs so slowly. We will begin assays aimed at answering this question in the next year.

Finally, we have begun our studies on the importance of the p68DDX5 RNA helicase in MMEC growth, demonstrating that established breast cancer lines require p68 expression to proliferate. Moreover, in primary MMECs, loss of p68 prevents their transformation by oncogenic Ras alleles, underscoring the overall importance of p68 expression in cellular transformation. We will build upon these results in the next year by generating a mouse model for p68 overexpression.

We have already generated a unique Arf knockout mouse model in the breast These will be invaluable resources for both the general breast cancer community and the future proposed studies in this grant.

REFERENCES

None

APPENDICES

None